

**REVIEW ARTICLE****Homocysteine and Thrombotic Disease**

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**H**OMOCYSTEINE is a non-protein-forming, sulfur amino acid whose metabolism is at the intersection of two metabolic pathways<sup>1</sup>: remethylation and transsulfuration (Fig 1). In remethylation, homocysteine acquires a methyl group from N-5-methyltetrahydrofolate (MTHF) or from betaine to form methionine. The reaction with MTHF occurs in all tissues and is vitamin B12-dependent, whereas the reaction with betaine is confined mainly to the liver and is vitamin B12-independent. A considerable proportion of methionine is then activated by adenosine triphosphate (ATP) to form S-adenosylmethionine (SAM). SAM serves primarily as a universal methyl donor to a variety of acceptors including guanidinoacetate, nucleic acids, neurotransmitters, phospholipids, and hormones. S-adenosylhomocysteine (SAH), the byproduct of these methylation reactions, is subsequently hydrolyzed, thus regenerating homocysteine, which then becomes available to start a new cycle of methyl-group transfer. In the transsulfuration pathway, homocysteine condenses with serine to form cystathionine in an irreversible reaction catalyzed by the pyridoxal-5'-phosphate (PLP)-containing enzyme, cystathionine  $\beta$ -synthase (CBS). Cystathionine is hydrolyzed by a second PLP-containing enzyme, gamma-cystathionase, to form cysteine and  $\alpha$ -ketobutyrate. Excess cysteine is oxidized to taurine and inorganic sulfates or excreted in the urine. Thus, in addition to the synthesis of cysteine, this transsulfuration pathway effectively catabolizes excess homocysteine which is not required for methyltransfer and delivers sulfate for the synthesis of heparin, heparan sulfate, dermatan sulfate, and chondroitin sulfate. It is important to note that because homocysteine is not a normal dietary constituent, the sole source of homocysteine is methionine.

Because of the existence of a cellular homocysteine export mechanism,<sup>2,3</sup> plasma normally contains a small amount of homocysteine averaging 10  $\mu$ mol/L. This export mechanism complements the catabolism of homocysteine through transsulfuration; together these mechanisms help maintain low intracellular concentrations of this potentially cytotoxic sulfur amino acid. In hyperhomocysteinemia, plasma homocysteine levels are elevated and, barring impaired renal function, the occurrence of hyperhomocysteinemia indicates that homocysteine metabolism has in some way been disrupted and that the export mechanism is disposing into the blood excess homocysteine. This export mechanism limits intracellular toxicity, but leaves vascular tissue exposed to the possibly deleterious effects of excess homocysteine.

**PATHOGENESIS OF HYPERHOMOCYSTEINEMIA**

The more severe cases of hyperhomocysteinemia are caused by homozygous defects in genes encoding for enzymes of homocysteine metabolism. In such cases, a defect in an enzyme involved in either homocysteine remethylation or transsulfuration leads to large elevations of homocysteine in the blood and urine. The classic form of such a disorder—congenital homocystinuria—is caused by homozygous defects in the gene encoding for CBS. In these individuals, fasting plasma homocysteine concentrations can be as high as 400  $\mu$ mol/L.<sup>4</sup> Two different forms of the disease can be distinguished on the basis of the responsiveness to treatment with large dosages of vitamin B6.<sup>5,6</sup> Several CBS mutations are known<sup>7-10</sup>: the most frequent are 833TC and 919GA, located in exon 8; and 1224-2AC, which causes the entirety of exon 12 being deleted. The 833TC is spread in several ethnic groups; the 919GA mutation has been almost exclusively reported in patients of Celtic origin. In 20 homocystinuric patients from 16 unrelated Italian families, characterization of 24 of 30 independent alleles disclosed 13 mutations, including 11 novel mutations (146CT, 172CT, 262CT, 346GA, 374GA, 376AG, 452del27, 770CT, 844ins68, 869CT, 904GA). Two previously reported mutations (833TC and 341CT) were found in 26.6% and 16.6% of the alleles. Hence, most of the mutations are 'private' and clustered on exons 8, 3, and 1.<sup>9,10</sup>

Homozygous defects of other genes that lead to similarly severe elevations in plasma homocysteine include those encoding for methylenetetrahydrofolate reductase (MTHFR) or for any of the enzymes which participate in the synthesis of methylated vitamin B12.<sup>11-17</sup> Genetic impairments for the

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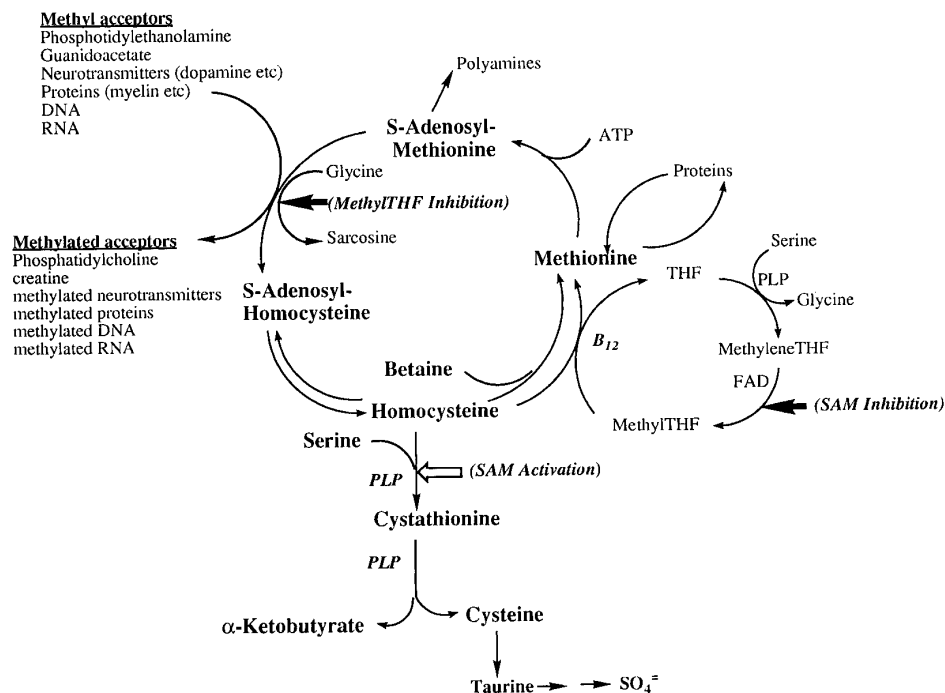
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**Fig 1. Homocysteine metabolic pathways.** In the methylation pathway, homocysteine acquires a methyl group either from betaine, a reaction that occurs mainly in the liver or from 5-methyltetrahydrofolate, a reaction which occurs in all tissues and is vitamin B<sub>12</sub>-dependent. In the transsulfuration pathway homocysteine condenses with serine to form cystathionine in a reaction that is catalyzed by CBS and requires PLP.

vitamin B<sub>12</sub>-dependent methyltetrahydrofolate:homocysteine methyltransferase have not been reported.<sup>4</sup> MTHFR deficiency was first described by Mudd et al<sup>14</sup> in two unrelated teenagers. In contrast to patients with CBS deficiency, these patients had slightly reduced methionine levels in their plasma and normal CBS activity in skin fibroblast extracts. Although the hyperhomocysteinemia associated with this defect is less severe than in homozygous CBS deficiency, the prognosis for these patients is generally worse than in CBS deficiency,<sup>18</sup> related in part to the unresponsiveness to any form of treatment. Most patients with MTHFR deficiency have hypomethioninemia, but in contrast to patients who have hypomethioninemia due to inborn errors of vitamin B<sub>12</sub> metabolism and who develop severe megaloblastic anemia,<sup>19</sup> patients with MTHFR deficiency do not become anemic. However, expression of MTHFR deficiency is variable<sup>14</sup> (see below).

Vitamin B<sub>12</sub>, an essential nutrient, is converted to methylcobalamin, which functions as a cofactor for methionine synthase, and to 5-deoxyadenosylcobalamin, the coenzyme for mitochondrial methylmalonyl-CoA mutase. Several distinct genetic defects of cobalamin transport and metabolism resulting in decreased methionine synthase activity and hyperhomocysteinemia have been described (transcobalamin II deficiency, Cbl C, D, E, and G mutations). These defects may selectively hamper methylation of vitamin B<sub>12</sub>. In addition, such defects may impair synthesis of 5-deoxyadenosylcobalamin, with resulting methylmalonic aciduria.<sup>19</sup>

The more common causes of hyperhomocysteinemia are also moderate in character and may be caused by less severe defects in genes encoding for enzymes or from inadequate status of those vitamins that are involved in homocysteine metabolism. Plasma homocysteine concentrations in these

instances may differ depending on which arm of the two metabolic pathways of homocysteine metabolism is defective.<sup>1</sup> An impairment in the remethylation pathway, even if it is mild, will lead to a substantial increase in plasma homocysteine concentrations under fasting conditions. Such an impairment may be due to inadequate status of either folate or vitamin B<sub>12</sub> or to defects in the gene encoding for MTHFR.<sup>1,17,20-33</sup> MTHFR contains flavin adenine dinucleotide (FAD) as a prosthetic group, which raises the possibility that vitamin B<sub>2</sub> status is also a determinant of fasting plasma homocysteine levels. In contrast, a mild impairment in the transsulfuration pathway will lead, at most, to a very slight increase in fasting plasma homocysteine levels. This mild impairment, which may be caused by heterozygous defects in the CBS gene or inadequate levels of vitamin B<sub>6</sub>,<sup>1,20,34-38</sup> is normally identified by an abnormal increase in plasma homocysteine after a methionine loading test or following a meal.<sup>20,38-41</sup> The different phenotype anticipated in remethylation and transsulfuration defects is supported by studies conducted in vitamin-deficient animal models. Thus, fasting plasma homocysteine concentration is 10-fold higher in folate deficient rats than in folate supplemented rats.<sup>42</sup> This high concentration of homocysteine in plasma was in part due to a lack of MTHF for homocysteine transmethylation and to insufficient S-adenosylmethionine for the activation of the transsulfuration pathway.<sup>42</sup> In both humans and rats, mild vitamin B<sub>6</sub> deficiency was associated with normal fasting plasma homocysteine levels. Fasting hyperhomocysteinemia in vitamin B<sub>6</sub> deficiency may occur only if the deficiency is severe and sustained over a long period of time.<sup>36</sup> After methionine load, the homocysteine concentration increased 35-fold in rats that were vitamin B<sub>6</sub>-deficient compared with about fourfold in control rats and less than

35% in folate-deficient rats.<sup>42</sup> Evidence of two distinct forms of hyperhomocysteinemia in humans is largely derived from preliminary data obtained from 274 consecutive participants in the Family Heart Study.<sup>43</sup> Plasma total homocysteine were measured under fasting conditions and 4 hours after a methionine load for each participant. Using homocysteine values greater than the 90th percentile for the definition of hyperhomocysteinemia (both fasting and methionine load), it was shown that of 43 hyperhomocysteinemic individuals, 20 (46%) had fasting hyperhomocysteinemia only, 17 (34.5%) had postmethionine load hyperhomocysteinemia only and just 7 (14%) had both types of hyperhomocysteinemia.

Existence of an interrelationship between vitamin status and plasma homocysteine was first reported by Kang et al,<sup>17</sup> who showed an inverse relationship between homocysteine and plasma folate concentrations. Other studies have shown the existence of an inverse correlation between homocysteine and folate or vitamin B12 plasma concentrations and the efficacy of vitamin supplementation in the lowering of plasma homocysteine levels.<sup>22-24,26-31,33-35,44-51</sup> The independent associations between individual nutrients and homocysteine concentrations were studied in an established cohort of Americans<sup>52</sup> (The Framingham Heart Study). After controlling for age, sex, and other vitamins, nonfasting plasma homocysteine exhibited a strong, nonlinear inverse association with plasma folate. Minimum levels of homocysteine were observed around 10 nmol/L of folate and above. Nonfasting plasma homocysteine exhibited weaker inverse associations with plasma concentrations of vitamin B12 and pyridoxal-5'-phosphate (PLP, the active form of vitamin B6).

Potential interactions between vitamin status and genetic abnormalities of the enzymes involved in methionine metabolism in the pathogenesis of hyperhomocysteinemia is illustrated by the recent data on the thermolabile MTHFR, a variant of the enzyme which is caused by a mutation in the structural gene at a polymorphic site (see below).

#### INTERACTION BETWEEN MTHFR THERMOLABILITY AND VITAMIN STATUS

Mutations that result in severely reduced MTHFR activity and hyperhomocysteinemia are rare.<sup>53-55</sup> However, in 1988 Kang et al<sup>17</sup> reported that two unrelated patients with moderate hyperhomocysteinemia and low folate levels had a variant of MTHFR that was distinguished from the normal enzyme (as measured in lymphocyte extracts) by its lower specific activity (50%) and its thermolability. In subsequent studies, Kang et al<sup>56,57</sup> showed that MTHFR thermolability is an inherited recessive trait, which is present in approximately 5% of the general population and 17% of patients with proven coronary artery disease, but is not associated with neurological complications. Impaired activity of MTHFR, due to the thermolabile form of the enzyme, has been observed in as many as 28% of hyperhomocysteinemic patients with premature vascular disease.<sup>58</sup> The cDNA for human MTHFR has been recently isolated<sup>54</sup> and it has been shown that MTHFR thermolability is caused by a point mutation (677C to T transition) at a polymorphic site, resulting in a valine substitution for an alanine in this enzyme.<sup>59</sup> The mutation was found in 38% of unselected chromosomes from

57 French Canadian individuals; the homozygous state for the mutation was present in 12% of these subjects and correlated with significantly raised tHcy.<sup>59</sup> Preliminary evidence indicates that the frequency of homozygotes for the 677CT mutation may vary significantly in populations from different geographic areas (from 1.4% to 15%).<sup>60</sup> In the Dutch population, homozygosity for this mutation is 15% and 5% in 60 vascular patients and 111 controls, respectively.<sup>61</sup> In an Italian patient population with arterial or venous occlusive disease, the prevalence of homozygotes was 19 of 64 individuals (29.7%).<sup>62</sup> In a control group (n = 258), on the other hand, homozygosity for the 677CT mutation was only 15.1%. Interestingly, none of 7 patients with isolated methionine intolerance was homozygous for the mutation.<sup>63</sup>

The impact of MTHFR thermolabile variant on plasma homocysteine levels is as of yet unclear. The hyperhomocysteinemia seen in the original patients of Kang et al<sup>17</sup> was associated with low folate plasma levels, and folate supplementation reduced homocysteine to normal levels. In the above-mentioned Italian study,<sup>62</sup> 18 of the 19 patients who were homozygotes for the 677CT mutation were hyperhomocysteinemic with levels of homocysteine in plasma that were higher than the 95th percentile in the respective age-matched controls. In a recent study the occurrence of an interaction between MTHFR thermolability genotype and folate status was shown.<sup>64</sup> When plasma folate concentrations were above the median (15.4 nmol/L), plasma homocysteine levels were low and unrelated to the MTHFR genotype. However, when plasma folate concentrations were below the median, plasma homocysteine levels were significantly higher in homozygotes for the 677CT mutation than in those with the normal genotype.<sup>64</sup> These data imply that the phenotypic expression of the MTHFR genotypes is dependent on the availability of folate, suggesting that homozygotes for the thermolabile genotype might have a higher folate requirement than individuals with a normal genotype. Because FAD is an essential prosthetic group for MTHFR activity, it stands to reason that vitamin B2 status is also a determinant of plasma homocysteine levels. The latter relationship has yet to be demonstrated in population-based studies.

#### HYPERHOMOCYSTEINEMIA AND THROMBOEMBOLIC DISEASE

The relationship between severe hyperhomocysteinemia and arterial disease was first suggested by McCully,<sup>65</sup> who observed autopsy evidence of precocious arterial thrombosis and atherosclerosis in a homocystinuric patient with impaired cobalamin metabolism that was identical to what had earlier been described in homocystinuric patients with CBS deficiency.<sup>66</sup> This deficiency in CBS is characterized by arteriosclerosis, thromboembolic complications, skeletal abnormalities, ectopia lentis, and mental retardation. In 1976, Wilcken and Wilcken<sup>67</sup> showed that the concentration of homocysteine-cysteine mixed disulfide after a methionine load was slightly higher in coronary heart disease patients than in controls, thus providing the first evidence of an association between mild hyperhomocysteinemia and vascular disease. Mildly increased homocysteine levels were later reported in coronary artery disease patients,<sup>68-73</sup> in cerebrovas-

Table 1. Relationship Between Homocysteine and Thrombosis

Authors	Thrombosis Type	Age (yr)	Homocysteine Measured	Homocysteine ( $\mu\text{mol/L}$ )		Hyperhomocysteinemia Cases		
				Patients (n)	Controls (n)	Patients (%)	Controls (%)	OR (95% CI)
Brattstrom et al <sup>92</sup>	Venous	<50	Fasting	13.4 (29)†	10.6 (30)			
			PML ( $\Delta$ )*	23.2†	20.4			
			PML	(42)‡	(42)	14.5	5	3.1 (0.4, 27.7)
Amundsen et al <sup>93</sup>	Deep venous	<56	Fasting	10.3 $\pm$ 3.5 (35)	10.3 $\pm$ 2.6 (39)	5.7	2.6	2.3 (0.1, 68.5)
			PML(T)*	39.5 $\pm$ 14.2	37.2 $\pm$ 14.2	5.7	2.6	2.3 (0.1, 68.5)
Bienvenu et al <sup>94</sup>	Venous + arterial	<58	Fasting	13.8 $\pm$ 6.0 (50)	8.1 $\pm$ 2.2 (49)	36	4	13.2 (2.82, 83.0)
Falcon et al <sup>95</sup>	Juvenile venous	<40	Fasting	7.4 $\pm$ 5.5 (80)	7.9 $\pm$ 2.1 (51)	8.8	0	$\infty$ (1.16, $\infty$ )
			PML( $\Delta$ )*	10.3 $\pm$ 5.1 (79)	8.3 $\pm$ 3.4 (40)	18.8	2.5	9.1 (1.38, 194)
Fermo et al <sup>96</sup>	Venous + arterial	<45	Fasting	14.7 $\pm$ 9.1 (157)	11.6 $\pm$ 3 (60)	8.9	5	1.9 (0.5, 6.6)
			PML(T)*	25.6 $\pm$ 11.6 (87)	19.3 $\pm$ 7.1 (60)	21.8	5	5.3 (1.7, 17.1)
den Heijer et al <sup>97</sup>	Deep vein	44	Fasting	12.9 (269)	12.3 (269)	10	5	2.5 (1.2, 5.2)
Cattaneo et al <sup>98</sup>	Deep vein	N/A	Fasting + PML(T)	N/A (89)	N/A (89)	13.5	6.7	2.2 (0.8, 6.0)
Simioni et al <sup>99</sup>	Deep vein	62	Baseline	15 (60)	12 (148)	25	11.5	2.6 (1.1-5.9)
den Heijer et al <sup>100</sup>	Recurrent thrombosis	67P 51C	Fasting	N/A (185)	N/A (220)	25	10	2.0 (1.5, 2.7)
			PML(T)			24	10	2.6 (1.9, 3.6)
Petri et al <sup>101</sup> §	Atherothrombotic lupus	38	Fasting	N/A (337)		15	10	3.49 (0.97, 12.54)

Abbreviation: N/A, data not available.

\* T, total plasma homocysteine level after methionine load (fasting homocysteine level was not subtracted).  $\Delta$ , Net increase in homocysteine after methionine load; ie, total fasting levels.

† Males only.

‡ Males and females.

§ Prospective study.

cular disease patients,<sup>69,74-78</sup> and in peripheral artery disease patients.<sup>69,75,79-81</sup> Association of mild hyperhomocysteinemia with occlusive disease<sup>76,77,80,82-86</sup> was independent of the presence of risk factors like smoking, hyperlipidemia, hypertension, and diabetes. This suggested that hyperhomocysteinemia is an independent risk factor for the abovementioned clinical conditions. Homocysteine concentrations in patients with symptomatic vascular disease are on average 31% greater than in normal controls,<sup>87</sup> and prospective assessment of vascular disease risk indicates that plasma homocysteine levels are associated with increased risk of myocardial infarction.<sup>88,89</sup>

Although venous thromboembolism accounts for 50% of the vascular complications of homocystinuria,<sup>90</sup> the link between less severe hyperhomocysteinemia and venous thromboembolic disease was overlooked until recently. Recurrent episodes of thromboembolism, events that occur at an early age, thrombosis after trivial provocation, and thrombosis at unusual sites are all features which should heighten the suspicion that an inherited metabolic abnormality is playing an etiologic role.<sup>91</sup> Studies on the relationship between thrombosis and mild hyperhomocysteinemia are summarized in Table 1. Brattstrom et al<sup>92</sup> found no significant difference in plasma homocysteine concentrations between 42 patients with venous thromboembolism and healthy control subjects, although the male patients showed a tendency of higher plasma homocysteine than male control subjects. Similarly,

Amundsen et al<sup>93</sup> studied 35 young adult patients (age <56 years) with verified deep venous thrombosis and found no difference in fasting and postmethionine load plasma homocysteine levels with age/sex-matched healthy controls. However, other investigators have reported a positive association between hyperhomocysteinemia and venous thrombotic outcomes. In one study<sup>94</sup> elevated fasting plasma homocysteine levels were reported in 25% of patients who developed venous thrombosis before 60 years of age. Fasting and postmethionine load homocysteine levels were measured by Falcon et al<sup>95</sup> in a series of 80 patients who had at least one verified episode of venous thromboembolism before the age of 40 years and were free from hemostatic abnormalities known to be associated with increased risk of venous thromboembolism. Fasting hyperhomocysteinemia was observed in 8.8% of patients, but postmethionine load hyperhomocysteinemia was present in 17.7% of the patients. About half of the patients with hyperhomocysteinemia had a positive family history of thrombosis and familial hyperhomocysteinemia was confirmed in over 50% of the families studied. In a cross-sectional 2-year evaluation of 157 consecutive unrelated patients with a history of venous or arterial occlusive disease occurring before the age of 45 years or at unusual sites, moderate hyperhomocysteinemia was detected in 13.1% and 19.2% of patients with venous or arterial occlusive disease.<sup>96</sup> The prevalence of hyperhomocysteinemia was almost twice as high when based on homocysteine measure-

ments done after oral methionine load as when based on fasting levels. Deficiencies of protein C, protein S, plasminogen, and activated protein C resistance were detected only in patients with venous occlusive disease, with an overall prevalence of 18.7%. Familial hyperhomocysteinemia was shown in 8 of the 12 families investigated. Event-free survival analysis showed that the relative risk in patients with moderate hyperhomocysteinemia and the other defects was 1.7 times greater than in patients without defects and that the risk conferred by hyperhomocysteinemia was similar to that of defects affecting the protein C system. A higher rate of recurrent thrombosis was also observed in patients with hyperhomocysteinemia and with the other defects than in patients without defects.<sup>97</sup> Homocysteine levels above the 90th percentile of the control distribution were observed in another study in 25% of 185 patients with recurrent venous thrombosis, with a relative risk of recurrence two times greater in patients with hyperhomocysteinemia than in those without hyperhomocysteinemia.<sup>97</sup> In this study, the relative risk of patients with postmethionine load homocysteine concentrations exceeding the 90th percentile (2.6) was similar to that of patients with fasting hyperhomocysteinemia. Twenty-seven of the 46 patients with fasting hyperhomocysteinemia also had postload hyperhomocysteinemia, whereas 17 patients had isolated methionine intolerance. Hence, the overall prevalence of hyperhomocysteinemia in this patients' population was 34.1%. However, because absolute postmethionine load values (instead of the postmethionine load above baseline levels) were considered in this study, the relative contribution of remethylation or transsulfuration defects in the risk conferred by hyperhomocysteinemia could not be evaluated.

These data represent strong evidence supporting the role of moderate hyperhomocysteinemia in the development of premature and/or recurrent venous thromboembolic disease. High plasma homocysteine levels are also a risk factor for deep-vein thrombosis in the general population. Fasting homocysteine concentrations were measured in 269 patients below 70 years of age with a first episode of deep-vein thrombosis and matched control subjects participating in the Leiden Thrombophilia Study.<sup>100</sup> Hyperhomocysteinemia exceeding the 95th percentile of the control group was found in 10% of the patients, with a matched odds ratio of 2.5. The effect of hyperhomocysteinemia was independent of other well-established risk factors for thrombosis, including protein C, protein S, and antithrombin III deficiencies and activated protein C resistance. An unexpected finding of this study was the observation that the association between elevated homocysteine levels and venous thrombosis was stronger among women than among men. Because nutritional parameters were not evaluated in this study, it cannot be ruled out that the stronger association observed in women may be caused by a different vitamin status. In addition, postmethionine load homocysteine measurements were not carried out, resulting in a potential underestimation of the risk conferred by hyperhomocysteinemia.

Most recently, Petri et al<sup>101</sup> reported a prospective study on the association between homocysteine and risk of stroke and thrombotic events in 337 systemic lupus erythematosus

(SLE) patients with follow-up of 1,619 person-years (mean 4.8 [SD 1.7] years). A fasting blood sample was obtained at the beginning of this study from each patient who also had four follow-up assessments per year to establish risk factors for thrombosis and coronary artery disease. During the follow-up there were 29 cases of stroke and 31 arterial thrombotic events. Hyperhomocysteinemia, defined as total plasma homocysteine  $>14.1 \mu\text{mol/L}$ , was found in 15% of these patients and was significantly associated with arterial thrombotic events (odds ratio 3.74 [95% confidence interval (CI) 1.96 to 7.13],  $P = .0001$ ) and with stroke (odds ratio 2.24 [95% CI 1.22 to 4.13],  $P = .01$ ). After adjustment for established risk factors, hyperhomocysteinemia remained an independent risk factor for thromboses ( $P = .05$ ) and stroke ( $P = .04$ ).

As in the case for deficiencies of the protein C anticoagulant system, not all patients with hyperhomocysteinemia develop thrombosis. The possibility that factors synergistic to hyperhomocysteinemia may be required for the development of thrombotic manifestations was explored in 45 members of seven unrelated consanguineous kindreds in which at least one member was homozygous for homocystinuria.<sup>101</sup> Thrombosis occurred in 6 of 11 patients with homocystinuria before the age of 8 years; all 6 patients also had activated protein C resistance. Conversely, of four patients with homocystinuria who did not have activated protein C resistance, none had thrombosis occurring before the age of 17 years. The investigators concluded that the combination of homocystinuria and activated protein C resistance conveys a substantial risk for thrombosis.<sup>102</sup> Such a conclusion may cast doubts about an independent pathogenic role of hyperhomocysteinemia in venous thromboembolism. Both activated protein C resistance and hyperhomocysteinemia are highly prevalent in patients with early onset vascular occlusive disease.<sup>96</sup> If the association of activated protein C resistance and moderate hyperhomocysteinemia markedly increases the thrombotic risk, one would anticipate its prevalence to be significantly higher than expected based on the prevalence of the isolated defects. In a series of 307 patients with early onset venous or arterial disease or with thrombosis occurring at unusual sites, the prevalence of isolated activated protein C resistance and moderate hyperhomocysteinemia (fasting or postmethionine-load) were 10% and 27%, respectively. The combined defect was detected in 3.6% of the patients, a figure slightly, but not significantly, higher than the 2.7% prevalence expected, assuming no effect of the association on the risk of thrombosis.<sup>103</sup> Although other possibilities cannot be ruled out, these data are consistent with the notion that moderate hyperhomocysteinemia, per se, is an independent risk factor for both venous and arterial thromboembolic disease.

#### THROMBOGENIC MECHANISMS OF HYPERHOMOCYSTEINEMIA

Despite the large number of studies suggesting that mild elevations of homocysteine in plasma are associated with an increased risk for occlusive vascular disease, thrombosis, and stroke, the question of whether homocysteine, per se, is responsible for these associations remains uncertain. The survey of Mudd et al,<sup>104</sup> which assessed the parents and

grandparents of homocystinuric children, concluded that heterozygosity for CBS deficiency is not associated with increased risk in heart attacks and stroke. Swift and Morrell<sup>105</sup> questioned the validity of some of the methods used by Mudd et al and argued that the data actually point to increased mortality rates in this heterozygote population. Two studies that used a noninvasive (doppler ultrasound) technique also provided conflicting results. Clarke<sup>106</sup> found no evidence of increased frequency of endothelial plaques in the neck arteries of 25 Irish heterozygotes, compared with 21 control subjects. Rubba et al,<sup>84</sup> on the other hand, indicated more frequent early vascular lesions in the iliac and internal carotid arteries in 14 heterozygotes than in 47 controls. At the molecular biology level, Kozich et al<sup>107</sup> examined the CBS alleles in four patients with premature occlusive arterial disease who were (1) hyperhomocysteinemic based on postmethionine load results, and (2) had lower enzyme activity in their fibroblasts. None of the eight alleles contained any mutation which resulted in diminished enzyme activity. In a prior study this group of investigators showed that cultured fibroblasts are not always reliable for testing the phenotypic expression in homocystinuric patients.<sup>7</sup>

Other studies<sup>61</sup> found that prevalence of the more common CBS mutations is not higher in the patient populations. A knockout mouse with CBS deficiency reported by Watanabe et al<sup>108</sup> was found to lack manifestations of thrombosis or/and cardiovascular complications and instead exhibited fatty livers. This is despite the fact that the levels of homocysteine in plasma were 40-fold higher in the homozygote mice than in the control mice. It might be that these mice did not live long enough for cardiovascular complications to develop.

Other inconsistencies relate to the question of whether MTHFR thermolability confers increased risk for the various diseases. The early study by Kang et al,<sup>57</sup> who relied on enzyme activity, has shown a higher prevalence (17%) of this variant in a North American, coronary artery disease (CAD) population than in normal healthy controls (5%). Similarly, the abovementioned studies, which relied on molecular biology techniques, demonstrated higher prevalence of the 677CT homozygotes in vascular disease patients (15%) than in controls (5%) in a Dutch population; and to 29.7% in an Italian patient population, compared with only 15.1% in healthy controls.<sup>61,62</sup>

However, other studies have been contradictory. No difference in the prevalence of the homozygosity for the 677CT mutation was found between coronary patients and controls in an Australian population,<sup>109</sup> a second Dutch population,<sup>110</sup> a French Canadian population,<sup>111</sup> a British population,<sup>112</sup> and in the Physician Health Study.<sup>113</sup>

These apparent inconsistencies can be explained on the basis of the differences in the study population, both with respect to genetic background and dietary habits, differences in the pathology among species (eg, human beings *v* mice), and others. It is for these reasons that understanding the mechanism that underlies this relationship between homocysteine and disease is of primary importance. What follows is a brief summary of this effort.

Early animal studies suggested a toxic effect of hyperhomocysteinemia on endothelial cells, resulting in shortened

platelet survival,<sup>114</sup> but these data have not been confirmed.<sup>115,116</sup>

In vitro studies of cultured endothelial cells also showed a toxic effect of homocysteine on cell viability and function, but these studies were conducted using extremely high homocysteine concentrations (1 to 10 mmol), exceeding the levels encountered even under the most severe pathologic conditions.<sup>114,117-119</sup> Furthermore, at no time did these studies show that the observed effect was specific for homocysteine and cannot be observed with other sulfur-containing compounds, particularly cysteine, whose concentration in plasma is 20- to 25-fold higher than that of homocysteine. Nonspecific inhibition of prostacyclin synthesis<sup>120</sup> and activation of factor V<sup>121</sup> by high concentrations of homocysteine on cultured endothelial cells has been reported. Inhibition of protein C activation<sup>122</sup> and downregulation of thrombomodulin expression<sup>123</sup> at homocysteine concentrations greater than 5 mmol/L have been also observed. One to 5 mmol homocysteine specifically blocks t-PA, but not plasminogen binding to endothelial cells.<sup>124</sup> The toxic effect of high homocysteine concentrations on endothelial cells<sup>125</sup> also results in increased platelet adhesion,<sup>120</sup> impaired regulation of endothelium-derived relaxing factor and related nitrogen oxides,<sup>126</sup> induction of tissue factor,<sup>127</sup> suppression of heparan sulfate expression,<sup>128</sup> and stimulation of smooth muscle cell proliferation.<sup>129</sup> Hyperhomocysteinemia may induce oxidation of low-density lipoprotein in vitro.<sup>130</sup> Because homocysteine can participate in disulfide bond exchange reactions, it is possible that excessive homocysteine entering the circulation can alter plasma proteins by this process. It has been reported that homocysteine concentrations as low as 8  $\mu$ mol/L markedly increases the affinity of Lp(a) for plasmin modified fibrin surfaces, thus inhibiting plasminogen activation.<sup>131</sup>

It is generally held that different mechanisms are responsible for arterial and venous thromboembolic diseases, involving platelet function abnormalities in arterial thrombosis and abnormalities of coagulation and/or fibrinolysis in venous thromboembolism. Ex vivo studies looking for such abnormalities in patients with hyperhomocysteinemia have given inconclusive results.<sup>115,116,132-137</sup> In subjects with severe hyperhomocysteinemia caused by homozygous CBS deficiency, an abnormally high in vivo biosynthesis of thromboxane A<sub>2</sub>—as reflected by the urinary excretion of its major metabolite 11-dehydro-thromboxane B<sub>2</sub>—has been observed.<sup>138</sup> Administration of aspirin inhibits thromboxane production; urinary appearance returns to baseline levels over a time course consistent with platelet survival, suggesting platelets are the major source of increased thromboxane urinary excretion.<sup>138</sup> Because thrombin is a potent inducer of platelet activation, the presence of hypercoagulable state was investigated in homocystinuric patients.<sup>139</sup> Increased levels of prothrombin fragment 1.2, thrombin-anti-thrombin complex, and activated protein C were all observed in homocystinuric patients on vitamin treatment who were free of vascular disease. However, these abnormalities did not correlate with urinary thromboxane excretion. Interestingly, protein C levels, but not factor VII and factor II levels, were significantly reduced in homocystinuric patients and correlated with the degree of hyperhomocysteinemia.<sup>139</sup>

Diet-responsive deficiency of factor VII was previously reported in CBS-deficient patients.<sup>134,135,140</sup> Reduced protein C levels may at least partly contribute to venous thrombotic manifestations of patients with homozygous CBS deficiency. The observation that the increased urinary thromboxane excretion was independent of homocysteine levels and was present both in vitamin B6-responsive and nonresponsive patients may have an impact on treatment of hyperhomocysteinemia.<sup>139</sup> It is noteworthy that although the effectiveness of vitamin B6 in preventing thromboembolism in pyridoxine-responsive patients was shown to be statistically highly significant, the occurrence of thromboembolism was not abolished by vitamin supplementation.<sup>19</sup>

A recent study by Lentz et al<sup>141</sup> used a cynomolgus monkey model to investigate possible mechanisms of action of mild hyperhomocysteinemia. Mild hyperhomocysteinemia was induced by a diet high in methionine, depleted of folate, and free of choline. Total homocysteine concentrations were 10.6  $\mu\text{mol/L}$  in the experimental monkeys and 4.0  $\mu\text{mol/L}$  in the control group. In response to activation of platelets by infusion of collagen, blood flow to the leg decreased by 42% compared with 14% in controls. The response of resistance vessels to the endothelium-dependent vasodilators, adenosine diphosphate (ADP) and acetylcholine, were markedly impaired in hyperhomocysteinemic monkeys, which indicates that increased vasoconstriction in response to collagen may be caused by decreased vasodilator responsiveness to platelet-generated ADP. Furthermore, thrombomodulin anticoagulant activity in aorta decreased by 34% in the hyperhomocysteinemic monkeys.

Another recent study with promising results showed that incubation of aortic endothelial cells with 50  $\mu\text{mol/L}$  homocysteine for 4 hours led to 64% reduction in the level of glutathione peroxidase.<sup>142</sup> Glutathione peroxidase is a key enzyme in the oxidative defense mechanism which was shown to potentiate the action of nitric oxide. These observations indicate that homocysteine may cause endothelial cell injury both by promoting the formation of peroxides as well as by impairing their inactivation. Potential antagonism between nitric oxide (NO)-related functions and homocysteine were first reported by Stamler et al<sup>126</sup> and recently discussed in an editorial by Loscalzo,<sup>143</sup> who regards this antagonism as stemming from a deleterious effect of chronic exposure to hyperhomocysteinemia on the production of NO<sup>+</sup>, which ultimately leads to unopposed homocysteine-mediated oxidative injury to the endothelium. Existence of antagonism between homocysteine and NO was also implied in a more recent study by de Groote et al,<sup>144</sup> which showed that the virulence of *Salmonella typhimurium* in mice was attenuated in bacterial strains that were incapable of synthesizing homocysteine. Homocysteine may act as NO<sup>+</sup> receptor from S-nitrosothiol NO-donor compounds, thereby antagonizing the effect of NO in diverse processes including infection, atherosclerosis, and neurologic disease. Although further experiments are required, these data point to the possibility that elevated homocysteine exerts its effect on more than one system.

#### TREATMENT OF HYPERHOMOCYSTEINEMIA

Elevations in plasma homocysteine are common in the general population, particularly in the elderly. Vitamin status

is a primary determinant of mild to moderate hyperhomocysteinemia accounting for approximately two thirds of all such cases.<sup>50</sup> Vitamin supplementation results in near normalization of plasma homocysteine in most cases.<sup>145,146</sup> A recent meta-analysis of 38 studies evaluated the risk of hyperhomocysteinemia for arteriosclerotic vascular disease, estimated the reduction of homocysteine levels by folic acid administration, and calculated the potential reduction of coronary artery disease mortality by increasing folic acid intake.<sup>147</sup> These analyses proposed that elevations of total homocysteine were an independent graded risk factor for arteriosclerotic vascular disease and calculated that folic acid fortification of food would reduce the annual mortality by 50,000. Vitamin supplementation may also reduce recurrence of venous thromboembolic disease in patients with hyperhomocysteinemia. However, at present the clinical efficacy of this approach has not been tested. In addition, the bulk of evidence indicates that fasting total homocysteine determinations may identify up to 50% of the total population of hyperhomocysteinemic subjects. Patients with isolated methionine intolerance may benefit from vitamin B6 supplementation.

The time is ripe for a placebo/controlled multicenter trial for determining the efficacy of vitamin supplementation in the reduction of morbidity and mortality among patients with occlusive vascular disease, stroke, and thrombosis. Because vitamins are relatively inexpensive, there is little incentive from the part of drug companies to support such a trial and it is up to government agencies to assume this task. For these reasons it is important that the design of such a trial take into account all the information available. Homocysteine metabolism requires the participation of folate as well as vitamin B12 and vitamin B6 coenzymes. Reduction of homocysteine levels in plasma requires that all three of these vitamins are supplemented.

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